Biotechnol Lett (2006) 28:1761–1765
DOI 10.1007/s10529-006-9153-0

ORIGINAL PAPER

# Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis*

**Kenneth M. Bischoff · Alejandro P. Rooney · Xin-Liang Li · Siqing Liu · Stephen R. Hughes**

Received: 20 April 2006 / Accepted: 1 July 2006 / Published online: 10 August 2006


**Abstract** Strains of thermophilic bacilli were screened for cellulolytic activity by gel diffusion assay on selective medium at 55°C. Strain B-41361, identified as a strain of *Bacillus licheniformis*, displayed activity against carboxymethylcellulose. Zymogram analysis demonstrated several catalytically active polypeptides with the most prominent species having a mass of 37 kDa. The enzyme was purified 60-fold with a 17% yield and specific activity of 183 U/mg. The amino terminal sequence was homologous to members of glycoside hydrolase family 5. Optimal temperature was 65°C (measured over 30 min), but the enzyme was most stable at 60°C, retaining greater than 90% activity after one hour. The enzyme had a broad pH range, with maximal activity at pH 6.0, 75% maximal activity at pH 4.5, and 40% at pH 10. The enzyme hydrolyzed *p*-nitrophenylcellobioside, barley $\beta$-glucan, and lichenan, but no activity was detected against avicel or acid-swollen cellulose.

**Keywords** *Bacillus licheniformis* · Carboxymethylcellulase · Endoglucanase · Thermophilic

Mention of a trade name or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

K. M. Bischoff (✉) · A. P. Rooney · X.-L. Li · S. Liu · S. R. Hughes
U.S. Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, 1815 N. University St., Peoria, IL 61604, USA
e-mail: bischoffk@ncaur.usda.gov

## Introduction

Lignocellulosic biomass has the potential to replace starch as a feedstock for the production of fuel ethanol. But to make this feedstock economically competitive, efficient enzymatic processes that hydrolyze the cellulosic polysaccharides to fermentable sugars are needed. The term cellulase is loosely applied to enzymes that hydrolyze cellulose, but complete degradation of cellulose involves the action of three enzymatic activities (Lynd et al. 2002). Endoglucanases (EG) cleave the $\beta$-1,4-linkage in amorphous regions of polysaccharides to yield long-chain oligosaccharides. Cellobiohydrolases (CBH) cut in exo-fashion on oligomeric polysaccharides to yield cellobiose, a dimer of glucose. Finally, $\beta$-glucosidases hydrolyze cellobiose to glucose, which is then available for fermentation to fuel and other valuable products. Although many of the best-studied enzymes have come from cellulolytic fungi like *Trichoderma reesei*, thermophilic microorganisms may also serve as a

reservoir of robust hydrolytic enzymes that may be integrated into industrial fermentation processes (Stutzenberger 1991). We report here the screening of thermophilic bacilli for cellulolytic activity and the characterization of EG activity secreted from a thermophilic strain of *B. licheniformis*.

## Materials and methods

### Screening for cellulolytic and xylanolytic activity

Thermophilic bacterial strains were obtained from the ARS Culture Collection maintained at the USDA-ARS National Center for Agricultural Utilization Research, Peoria, IL. Liquid cultures were grown in shake flasks (150 rpm) at 55°C in medium containing the following per liter (Atlas 1993): Ten grams of tryptone, 5 g yeast extract, 2 g $K_2HPO_4$, pH 7.2. Bacteria were streaked on agar medium supplemented with either 0.1% (w/v) CMC or 0.1% (w/v) oat spelt xylan, and incubated at 55°C overnight. Plates were stained with Congo Red, destained with NaCl, and scored for a clear halo surrounding the colony (Wood et al. 1988).

### Preparation of crude cellulase by ammonium sulfate precipitation

Cultures were cleared of cells by centrifugation, and ammonium sulfate (516 g/l) was added to the cell-free culture supernatant to attain 75% saturation. Precipitated protein was collected by centrifugation at 20,000 × *g* for 20 min, and dissolved in PBS (140 mM NaCl, 12 mM sodium phosphate), pH 7.4, with PMSF at 1 mM. The solution was retained as the crude cellulase fraction and stored at 4°C. Protein concentration was determined by the method of Bradford against BSA as standard.

### Assay of enzymatic activity

Carboxymethylcellulase activity (CMCase) was measured using a modification of the dinitrosalicylic acid (DNS) assay described by Wood and Bhat (1988). Standard assays contained the following in 50 μl: 1% (w/v) CMC, 140 mM NaCl, 12 mM sodium phosphate buffer, pH 5.8, and diluted enzyme sample. After 30 min incubation at 55°C, reactions were terminated by addition of 200 μl DNS solution (10 g DNS/l, 16 g NaOH/l, and 300 g potassium sodium tartrate/l). For crystalline cellulose substrates, incubation times were extended to 18 h. After heating to 95°C for 10 min and cooling to 4°C for 10 min, absorbance at 540 nm was measured in a microplate reader. The enzymatic liberation of reducing sugar was determined against a glucose standard. One unit (U) of activity is defined as the amount of enzyme that produces one μmol reducing sugar per minute. Assays using *p*-nitrophenylcellobioside (pNPC) were performed by the method described by Saha (2004).

### Zymogram analysis

Crude cellulase samples (1 μg) were denatured by heating for 2 min at 95°C in 1% (w/v) SDS and 5% (v/v) β-mercaptoethanol, and applied to a 10% SDS-PAGE gel containing 0.1% (w/v) CMC polymerized within the gel matrix. Following electrophoresis, the gel was washed once for 20 min in 20% (v/v) isopropanol in PBS, pH 5.8, followed by three washes (20 min each) in PBS. The gel was incubated at 55°C in PBS for 1 h, stained with Congo Red (1 mg/ml) for 30 min, and destained with 1 M NaCl. EG activity was visible as clear bands against a red background.

### Hydrolysis of cello-oligosaccharides

Reactions contained 0.5% (w/v) oligosaccharide substrate and 32 ng enzyme in 40 μl PBS, pH 5.8. Following incubation at 55°C for 3 h, 1 μl aliquots were spotted on Whatman Partisil K6 TLC plates, and developed with three ascending elutions of acetonitrile/water (80:20, v/v). Sugars were detected by reaction with 0.2% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride in 3% (v/v) sulfuric acid (Bounias 1980).

## Results

### Identification of cellulase producing *B. licheniformis*

A screen of 47 strains of thermophilic bacteria that were originally deposited as *Bacillus stearothermophilus* in the ARS Culture Collection identified one strain that possessed cellulolytic activity on CMC plates (data not shown). This strain was moderately thermophilic, having a generation time of 22 min at 55°C compared to 41 min at 37°C. Glucose and xylose supported growth of this strain in minimal media grown aerobically, but the strain could not grow on CMC or crystalline cellulose as sole carbon source. Ribosomal DNA analysis of this strain indicated that it was related to *B. licheniformis*. The strain was designated as B-41361.

### Characterization of crude cellulase

Crude cellulase from strain B-41361 possessed hydrolytic activity on CMC (0.045 U/mg) and pNPC (0.054 U/mg). Addition of CMC and Sigmacell 50 to the growth medium did not significantly affect the specific activities of CMCase. Supplementation of media with 0.1% xylose, 0.1% glucose, or 4% glucose increased the specific activity of CMCase to 0.22, 0.2, and 0.54 U/mg, respectively.

Zymogram analysis indicated a prominent active polypeptide of mass 37 kDa in preparations of crude cellulase (Fig. 1). The CMCase activity in the crude cellulase fraction was optimally active at 55°C and pH 6.0, and retained 88% and 72% of its activity after incubation at 55°C for 18 h and 42 h, respectively. Stability decreased dramatically above 55°C, with a half-life of 36 min at 60°C and 12 min at 65°C.

### Purification and characterization of the 37 kDa EG

Table 1 shows a purification summary for the 37-kDa enzyme. N-terminal amino acid sequencing by automated Edman degradation (Midwest Analytical, Inc., St. Louis, MO) demonstrated that the enzyme was homologous to several

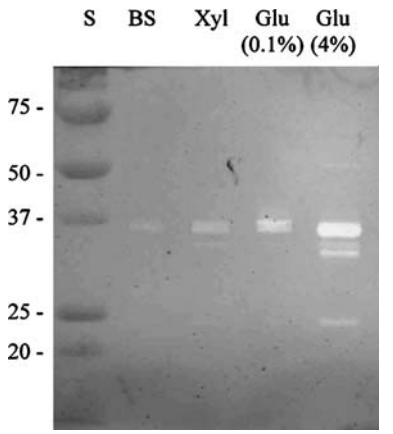


**Fig. 1** Zymogram analysis of crude cellulase fractions. Each lane contained 1 $\mu$g crude cellulase protein. Lane designations are as follows: S, BioRad Precision Plus Protein Standards; BS, culture grown in standard medium; Xyl, medium supplemented with 0.1% xylose; Glu (0.1%), medium supplemented with 0.1% glucose; Glu (4%), medium supplemented with 4% glucose. The mass of molecular markers (kDa) are listed on the left

Family 5 glycosyl hydrolases from other *Bacillus* species (Table 2). The purified CMCase was optimally active at 65°C and retained greater than 90% of its activity following incubation at 60°C for 1 h, but fell to 32% following incubation at 65°C. It had a broad pH activity profile, with a maximum at pH 6.0, but retaining 75% and 40% of maximal activity at pH 4.5 and 10, respectively. The enzyme hydrolyzed pNPC (34 U/mg), barley $\beta$-glucan (342 U/mg), and lichenan (187 U/mg), but no activity was detected against avicel, Sigmacell 50, acid-swollen cellulose, oat-spelt xylan, or *p*-nitrophenylglucoside. The enzyme hydrolyzed both cellopentaose and cellotetraose yielding G2 and G3 oligosaccharides (data not shown). A small G2 signal was visible in the products of cellotriose hydrolysis, but no hydrolysis of cellobiose was detected.

## Discussion

The amino terminus of the 37 kDa endoglucanase species of *B. licheniformis* strain B-41361 was identical to that predicted for the *bglc* gene of *B. licheniformis* ATCC 14580 and the *cel5A* gene from *B. licheniformis* strain GXN151, both of which are putative enzymes of glycosyl hydrolase

**Table 1** Purification summary of EG from *B. licheniformis* B-41361[a]

| Fraction | Volume (ml) | Activity (U) | Protein (mg) | Specific activity (U/mg) | Yield (%) | Enrichment (Fold) |
|---|---|---|---|---|---|---|
| Phenyl Sepharose | 22 | 6.5 | 2.1 | 3.1 | (100) | (1) |
| Desalted | 20 | 6.9 | 1.5 | 4.6 | 110 | 1.5 |
| High Q unbound | 22 | 2.2 | 0.03 | 73 | 34 | 24 |
| High S | 4 | 1.1 | 0.006 | 183 | 17 | 59 |

[a] Ammonium sulfate (164 g) was added to 1 l cell-free culture supernatant, and applied to a column of Phenyl Sepharose (1.5 × 10 cm) equilibrated in 30% (w/v) $(NH_4)_2SO_4$, 20 mM Tris, pH 7.5. Bound protein was eluted with 3 column volumes of a decreasing gradient from 30% to 0% (w/v) $(NH_4)_2SO_4$. Active fractions were combined as the Phenyl Sepharose fraction. The Phenyl Sepharose fraction was desalted by ultrafiltration and resuspension (2 times) using a 5000 MWCO ultrafiltration device and applied to a 5 ml High Q anion exchange column equilibrated in 20 mM Tris, pH 7.5. Unbound material from the High Q column was directly applied to a 5 ml High S cation exchange column equilibrated in the same buffer. Bound protein was eluted from the column with 5 column volumes of a linear gradient from 0 to 500 mM NaCl. Active fractions were combined as the High S fraction

**Table 2** Alignment of N-terminal sequence of B-41361 EG to Family 5 endoglucanases

| Species | Sequence |
|---|---|
| *B. licheniformis* B-41361 EG | 1 **ASKTPVAVNGQLTLKGTQLV** |
| *B. licheniformis* ATCC 14580 *bglC* | 50 **ASKTPVAVNGQLTLKGTQLV** |
| *B. licheniformis* GXN151 *cel5A* | 50 **ASKTPVAVNGQLTLKGTQLV** |
| *Bacillus* sp. NBL420 cellulase | 33 **AS**QT**PVAVNGQLTLKGTQLV** |
| *B. amyloliquefaciens* FZB42 *bglC* | 40 GT**KTPVA**K**NGQLTLKGTQLV** |
| *B. subtilis celC* | 31 GT**KTPVA**K**NGQL**SI**KGTQLV** |
| *Bacillus* sp. D04 *cel* | 31 GT**KTPVA**K**NGQL**SI**KGTQLV** |
| Consensus | +++TPVA NGQL++KGTQLV |

Accession numbers for the indicated sequences are as follows: *B. licheniformis* ATCC 14580 (YP079251; *B. licheniformis* GXN151 (AY291583.1; *Bacillus* sp. NBL420 (AAK73277.1; *B. amyloliquefaciens* FZB42 (CAE11243; *B. subtilis* (AY183475.1; *Bacillus* sp. D04 (I40548. Numbers to the left of the sequences indicate amino acid position in the published sequence. Bold type characters are used to indicate identity to the B-41361 sequence. A ''+'' in the consensus sequence denotes conservative substitutions across all sequences

family 5. Liu et al. (2004) reported the cloning of two endoglucanase genes from the Avicel degrading strain GXN151, *cel*9A and *cel*12A but zymogram analysis of crude cellulase from strain B-41361 did not indicate enzymes of equivalent masses to Cel9A and Cel12A. Strain B-41361 did not grow on CMC or crystalline cellulose, and the absence of detectable avicelase activity suggests that strain B-41361 does not possess a complete cellulase system, or degradation rates are too slow to support growth on cellulose substrates.

Detection of enzyme activity following SDS-PAGE may reveal the number of active polypeptides present in a biological sample along with an estimate of their relative abundance and approximate molecular weight (Bischoff et al. 1998). In the present study, the samples were completely denatured by treatment with detergent, reducing agent, and heat. Thus, the zymogram analysis should accurately reflect the molecular weight of the active monomer. Assuming an average mass of 110 g per amino acid, the predicted chain length of the 37 kDa endoglucanase is about 340 aa, considerably shorter than the related enzymes listed in Table 2. The *bglc* and *cel5A* gene products are 534 and 542 aa long, respectively, and both have putative C-terminal Family 3 carbohydrate binding modules (CBM) (Carbohydrate Active Enzymes database, available at http://afmb.cnrs-mrs.fr/CAZY/index.html). Based on size, the B-41361 enzyme presumably lacks a CBM, which would lower its affinity for crystalline cellulose and would be consistent with undetectable activity against this substrate (Carrard et al. 2000).

The B-41361 enzyme hydrolyzed cellotetraose to yield G2 and to a lesser extent G3 and G1, indicating that the first and/or third glycosidic

bond of cellotetraose can be cleaved by this enzyme. The enzyme also showed weak activity on G3. This suggests that the B-41361 enzyme acts in endo-fashion, but has broad substrate specificity for small chain oligosaccharides.

In conclusion, the predominant endoglucanase secreted by a thermophilic strain of *B. licheniformis* belongs to glycosyl hydrolase family 5. Its broad pH range and temperature stability may make it useful for industrial applications. Although characterizations of the native form purified from cell-free culture supernatants do not show activity against natural cellulose substrates, it may serve as a platform for directed mutagenesis to develop an enzyme for the efficient degradation of lignocellulosic biomass as feedstock for industrial fermentations.

**Acknowledgements** The authors wish to thank Eric Hoecker, Theresa Holly, Imran Khan, and Suzanne Platt for technical assistance.

## References

Atlas RM (1993) *Bacillus stearothermophilus* broth. In: Parks LC (ed) Handbook of Microbiological Media, CRC Press, Boca Raton, Florida, p 109

Bischoff KM, Shi L, Kennelly PJ (1998) The detection of enzyme activity following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal Biochem 260:1–17

Bounias M (1980) *N*-(1-Napthyl)ethylenediamine dihydrochloride as a new reagent for nanamole quantification of sugars on thin-layer plates by a mathematical calibration process. Anal Biochem 106:291–295

Carrard G, Koivula A, Soderlund H, Beguin P (2000) Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose. Proc Natl Acad Sci USA 97:10342–10347

Liu Y, Zhang J, Liu Q, Zhang C, Ma Q (2004) Molecular cloning of novel cellulase genes cel9A and cel12A from *Bacillus licheniformis* GXN151 and synergism of their encoded polypeptides. Curr Microbiol 49:234–238

Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577

Saha BC (2004) Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochem 39:1871–1876

Stutzenberger FJ (1991) Cellulose degradation by thermophilic aerobic bacteria. In: Haigler CH, Weimer PJ (eds) Biosynthesis and Biodegradation of Cellulose. Marcel Dekker, Inc. New York

Wood PJ, Erfle JD, Teather RM (1988) Use of complex formation between Congo Red and polysaccharides in detection and assay of polysaccharide hydrolases. Methods Enzymol 160:59–74

Wood TM, Bhat KM (1988) Methods for measuring cellulase activities. Methods Enzymol 160:87–112